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(71) Applicant (for all designated States except US): MERCK
& CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway,
NJ 07065-0907 (US).

L. [US/US]; 126 East Lincoln Avenue, Rahway, NJ
07065-0907 (US). JACOBS, Amanda, R. [US/US]; 126
East Lincoln Avenue, Rahway, NJ 07065-0907 (US).
KUO, Lawrence, C. [US/US]; 126 East Lincoln Avenue,
Rahway, NJ 07065-0907 (US).

(74) Common Representative: MERCK & CO., INC.; 126
East Lincoln Avenue, Rahway, NJ 07065-0907 (US).

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(72) Inventors; and

(75) Inventors/Applicants (for US only): DARKE, Paul,

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(54) Title: HEPATITIS C VIRUS REPLICATION INHIBITORS

(57) Abstract: The present application identifies the NS4A binding site present on NS2/3 as a target site for inhibiting NS2/3 protease activity. Methods inhibiting NS2/3 activity by targeting the identified target site are described along with examples of compounds useful in such methods and guidance for obtaining additional useful compounds. Such compounds and methods are preferably employed to inhibit HCV replication.

WO 01/16379 A1

TITLE OF THE INVENTION

HEPATITIS C VIRUS REPLICATION INHIBITORS

CROSS-REFERENCE TO RELATED APPLICATIONS

- 5 The present application claims priority to U.S. Serial No. 60/151,395, filed August 30, 1999, hereby incorporated by reference herein.

BACKGROUND OF THE INVENTION

- 10 The references cited herein are not admitted to be prior art to the claimed invention.

- 15 Hepatitis C virus (HCV) is a positive strand RNA virus that is the major cause of non-A, non-B hepatitis. (Choo, *et al.*, (1989) *Science* 244, 362-364; and Choo, *et al.*, (1989) *Science* 244, 359-362.) The HCV genome encodes a single polyprotein of approximately 3000 amino acids, containing the viral proteins in the order: C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B. The NS proteins are thought to be non-structural and are involved with the enzymatic functions of viral replication and processing of the viral polyprotein. Release of the individual proteins from the polyprotein precursor is mediated by both cellular and viral proteases. (Choo, *et al.*, (1991) *Proc. Natl. Acad. Sci. USA* 88, 2451-2455; Takamizawa, *et al.*, (1991) *J. Virol.* 65, 1105-1113; Neddermann, *et al.*, (1997) *Biol. Chem.* 378, 469-476; Lohmann, *et al.*, (1996) *J. Hepatol.* 24, 11-19; and Houghton, *et al.*, (1991) *Hepatology* 14, 381-388.)

- 25 The proteolytic release of mature NS4A, NS4B, NS5A and NS5B is catalyzed by the chymotrypsin-like serine protease contained within the N-terminal domain of NS3 (termed "NS3 protease"), while host cell proteases release C, E1, E2, and p7, and create the N-terminus of NS2 at amino acid 810. (Mizushima, *et al.*, (1994) *J. Virol.* 68, 2731-2734, and Hijikata, *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90, 10773-10777.)

- 30 The cleavage between amino acids 1026 and 1027 of the HCV polypeptide which separates NS2 from NS3 is dependent upon protein regions of both NS2 and NS3 flanking the cleaved site, and this autocleaving moiety is termed the NS2/3 protease. (Grakoui, *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90, 10583-10587; and Komoda, *et al.*, (1994) *Gene* 145, 221-226.) The cleavage is independent of the catalytic activity of the NS3 protease, as demonstrated with mutational studies.

(Grakoui, *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90, 10583-10587; and Hijikata, *et al.*, (1993) *J. Virol.* 67, 4665-4675.)

The NS2/3 cleavage reaction has been studied in bacterial, mammalian and insect cells, and following in-vitro translation of the protein. (Grakoui, *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90, 10583-10587; Selby, *et al.*, (1993) *J. Gen. Virol.* 74, 1103-1113; Hijikata, *et al.*, (1993) *J. Virol.* 67, 4665-4675; Santolini, *et al.*, (1995) *J. Virol.* 69, 7461-7471; D'Souza, *et al.*, (1994) *J. Gen. Virol.* 75, 3469-3476; and Pieroni, *et al.*, (1997) *J. Virol.* 71, 6373-6380.) The protein region essential for NS2/3 cleavage activity has been approximately mapped to amino acids 898 to 1207 of the HCV open reading frame. (Grakoui, *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90, 10583-10587; Hijikata, *et al.*, (1993) *J. Virol.* 67, 4665-4675; and Santolini, *et al.*, (1995) *J. Virol.* 69, 7461-7471.) The catalytic mechanism of NS2/3 cleavage is speculated to be either a metalloprotease or cysteine protease. (Wu, *et al.*, (1998) *Trends Biochem. Sci.* 23, 92-94; and Gorbalenya, *et al.*, (1996) *Perspect. Drug Discovery Design* 6, 64-86.) Cleavage activity of in-vitro translated NS2/3 is inhibited by EDTA and activity is restored with metal ion re-addition. (Hijikata, *et al.*, (1993) *J. Virol.* 67, 4665-4675; and Pieroni, *et al.*, (1997) *J. Virol.* 71, 6373-6380.)

NS4A is a cofactor for NS3 activity. The NS3 N-terminus that is formed by NS2/3 cleavage is markedly affected by association with the NS4A. Stable NS3-NS4 complex formation involves the N-terminal amino acid residues of NS3. (Sato, *et al.*, *J. Virol.* (1995) 69, 4255-4260.) The NS4A amino acid region primarily responsible for cofactor activity is located at about amino acids 22 to 31. (Shimizu, *et al.*, (1996) *J. Virol.* 70, 127-132; Butkiewicz, *et al.*, (1996) *Virology* 225, 328-338; and Lin, *et al.*, (1995) *J. Virol.* 69, 4373-4380.)

The X-ray crystallographic structure of NS3 bound to a peptide containing NS4A amino acids 21-34 (amino acids 1672-1685 of the HCV polyprotein) reveals that the NS3 N-terminal residues 2 through 9 directly interact with NS4A to compose one of 8 strands in an antiparallel beta-sheet extending through the NS3 protease. (Kim, *et al.*, (1996) *Cell* 87, 343-355; and Yan, *et al.*, (1998) *Protein Sci.* 7, 837-847, hereby incorporated by reference herein.) In contrast, without NS4A, the N-terminus of NS3 is poorly organized. (Love, *et al.*, (1996) *Cell* 87, 331-342.)

SUMMARY OF THE INVENTION

The present application identifies the NS4A binding site present on NS2/3 as a target site for inhibiting NS2/3 protease activity. Methods inhibiting NS2/3 activity by targeting the identified target site are described along with examples of compounds useful in such methods and guidance for obtaining additional useful compounds. Such compounds and methods are preferably employed to inhibit HCV replication or processing.

The "NS4A target site" refers to the NS4A binding site present on NS2/3. Proteinaceous and non-proteinaceous compounds can be used to target the NS4A target site. Preferred proteinaceous compounds are those containing a NS4A polypeptide region of about 11 contiguous amino acids that bind to the NS4A target site, variants of such compounds, prodrugs of such compounds, and pharmaceutical salts thereof. The region of NS4A, present in different HCV isolates, that binds to the target site is located at about amino acids 22-32.

Polypeptide regions of about 11 contiguous amino acids of NS4A that bind to the NS4A target site can readily be identified based upon the known NS4A amino acid sequences of different HCV isolates. Variations of such polypeptide regions can be obtained by substituting amino acids. Preferred substitutions are conservative substitutions and substitutions in those amino acids not essential for exerting an inhibitory effect on NS2/3 autocleavage.

Structure I provides a generic structure for polypeptides containing a region targeting the NS4A target site that can inhibit NS2/3 autocleavage. Structure I is as follows:

$Z^1-Y^1_m-X^1X^2X^3X^4X^5GX^6X^7X^8X^9X^{10}-Y^2_n-Z^2$

wherein X^1 is either serine, cysteine, or threonine;
 X^2 is either valine, leucine, or isoleucine;
 X^3 is either valine, leucine, isoleucine, serine, cysteine or threonine;
 X^4 is either valine, leucine, or isoleucine;
 X^5 is either valine, leucine, or isoleucine;
 X^6 is either lysine, arginine, or histidine;
 X^7 is either valine, leucine, or isoleucine;
 X^8 is either aspartic acid, glutamic acid, valine, leucine, isoleucine, lysine, arginine, or histidine;

X⁹ is either valine, leucine, or isoleucine;

X¹⁰ is either serine, cysteine, threonine, asparagine, glutamine, aspartic acid, or glutamic acid;

each Y¹ is an independently selected amino acid,

5 each Y² is an independently selected amino acid,

Z¹ is an optionally present protecting group covalently joined to Y¹,

Z² is an optionally present protecting group covalently joined to Y²,

m is from 0 to 300 and

n is from 0 to 300.

10 Preferred compounds can inhibit NS2/3 autocleavage at least about 50%, at least about 75%, at least about 85%, or at least about 95%; and/or have an IC₅₀ of at least about 5 μ M. Reference to "at least" with respect to IC₅₀ indicates potency. The ability of a compound to inhibit NS2/3 autocleavage is preferably measured using techniques such as those described in the Example section provided
15 below.

Thus, a first aspect of the present invention features a method of inhibiting HCV replication in an HCV infected cell using an effective amount of a compound that inhibits NS2/3 autocleavage. An effective amount to inhibit NS2/3 autocleavage is an amount that will cause a detectable reduction in NS2/3
20 autocleavage.

Another aspect of the present invention features a method of inhibiting HCV replication in an HCV infected cell using an effective amount of a nucleic acid comprising a nucleotide sequence encoding for (a) a polypeptide comprising an NS4A fragment at least about 11 amino acids in length or (b) a Structure I polypeptide. The
25 NS4A fragment is targeted to the NS4A target site and inhibits autocleavage of NS2/3. An effective amount to inhibit HCV replication is an amount that will cause a detectable reduction in HCV replication.

Nucleic acid comprising a nucleotide sequence encoding for a polypeptide can express the polypeptide inside a cell. Such nucleic acid can also
30 contain additional nucleotide sequences that may, for example, encode for other proteins.

A nucleotide sequence encoding for a polypeptide comprising an NS4A fragment at least about 11 amino acids in length encodes for at least 11 consecutive amino acids of an NS4A fragment. The polypeptide can contain

additional amino acid sequence regions present, or not present, in NS4A. Such additional regions should be selected so as not to reduce the ability of the polypeptide to exert its effect on HCV NS2/3 autocleavage. Examples of additional regions include those that remain part of the active polypeptide and those that are cleaved
5 inside a cell.

Another aspect of the present invention describes a method of treating a patient for HCV comprising the step of inhibiting NS2/3 autocleavage. Inhibiting HCV autocleavage is preferably performed using an effective amount of a compound that binds to the NS4A target site and reduces NS2/3 autocleavage.

10 A patient refers to a mammal undergoing treatment. A patient includes an individual being treated for an HCV infection or being treated prophylactically. Preferably, the patient is a human.

Another aspect of the present invention describes a method of inhibiting or preventing HCV replication in a patient comprising the step of treating
15 the patient with an effective amount of a compound containing a NS4A fragment at least about 11 amino acids in length or a Structure I polypeptide, a pharmaceutically acceptable salt of such a compound, or a prodrug thereof.

An effective amount to inhibit or prevent HCV replication is an amount that produces a detectable reduction in HCV replication in a patient infected
20 with HCV or confers to a patient the ability to resist HCV infection.

Another aspect of the present invention describes a method of inhibiting or preventing HCV replication in a patient comprising the step of administering to the patient an effective amount of a nucleic acid comprising a nucleotide sequence encoding for (a) a polypeptide comprising an NS4A fragment at
25 least about 11 amino acids in length or (b) a Structure I polypeptide. The NS4A fragment is targeted to the NS4A target site and inhibits autocleavage of NS2/3.

Another aspect of the present invention describes a compound that is either (1) an HCV inhibitor polypeptide comprising an NS4A fragment at least about 11 amino acids in length that can inhibit autocleavage of NS2/3; (2) a Structure I
30 polypeptide; (3) a pharmaceutically acceptable salt of (1) or (2); or (4) a prodrug of (1), (2), or (3); provided that if the compound is (1) or (2) then the compound contains either, or both, an amino protecting group or a carboxy protecting group.

Another aspect of the present invention features a nucleic acid comprising a nucleotide sequence encoding for a HCV inhibitor polypeptide

comprising either (a) an NS4A fragment at least about 11 amino acids in length that can inhibit autocleavage of NS2/3 or (b) a Structure I polypeptide.

Another aspect of the present invention describes a pharmaceutical composition for inhibiting HCV replication comprising a pharmaceutically acceptable carrier; and an effective amount of a compound that is either (1) an HCV inhibitor polypeptide comprising an NS4A fragment at least about 11 amino acids in length that can inhibit autocleavage of NS2/3; (2) a Structure I polypeptide; (3) a pharmaceutically acceptable salt of (1) or (2); or (4) a prodrug of (1), (2), or (3).

A pharmaceutically acceptable carrier refers to a carrier suitable for therapeutic administration that is combined with an active ingredient. The carrier itself is generally not active in treating or preventing a disease, but rather facilitates administration of the active ingredient. Examples of pharmaceutically acceptable carriers include calcium carbonate, calcium phosphate, lactose, glucose, sucrose, gelatin, vegetable oils, polyethylene glycols and physiologically compatible solvents. Additional examples, some of which are described below under "Administration", are well known in the art.

Another aspect of the present invention features a pharmaceutical composition for inhibiting HCV replication comprising a pharmaceutically acceptable carrier; and an effective amount of a nucleic acid encoding for a polypeptide that either (a) comprises a fragment of NS4A at least about 11 amino acids in length, wherein the fragment can inhibit autocleavage of NS2/3; or (b) is a Structure I polypeptide.

Another aspect of the present invention features a method for inhibiting HCV polyprotein processing comprising the step of contacting a cell expressing an HCV polypeptide that contains at least NS2/3 with an effective amount of a compound that is either (1) an HCV inhibitor polypeptide comprising an NS4A fragment at least about 11 amino acids in length that can inhibit autocleavage of NS2/3; (2) a Structure I polypeptide; (3) a pharmaceutically acceptable salt of (1) or (2); or (4) a prodrug of (1), (2), or (3).

Another aspect of the present invention features a method of screening for a compound that inhibits HCV replication or HCV polyprotein processing. The method is performed by (a) selecting for a compound that binds to the NS4A target site using a polypeptide comprising NS2/3 or a binding portion thereof, and (b) measuring the ability of the compound to inhibit HCV replication or HCV polyprotein processing.

“Comprising NS2/3 or a binding portion thereof” indicates that polypeptide regions from NS2 and NS3 needed for binding to NS4A are both present. Preferably, at least about 70 amino acids from the NS2 carboxy terminus are present and at least about 150 amino acids from the NS3 amino terminus are present in the NS2/3 portion.

HCV polyprotein processing refers to the formation of one or more HCV peptides. HCV polyprotein processing can be measured using different techniques such as by measuring the presence of an individual protein or the activity associated with an individual protein. Preferably, HCV processing is performed by measuring the activity or formation of NS2 or NS3.

Another aspect of the present invention features a method of screening for a compound that inhibits HCV replication or HCV polyprotein processing in the presence of a non-saturating amount of a NS4A agonist. A “NS4A agonist” is a compound that competes with NS4A for binding to NS2/3. The NS4A agonist also inhibits, to some extent, NS2/3 autocleavage.

Other features and advantages of the present invention are apparent from the additional descriptions provided herein including the different examples. The provided examples illustrate different components and methodology useful in practicing the present invention. The examples do not limit the claimed invention. Based on the present disclosure the skilled artisan can identify and employ other components and methodology useful for practicing the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates HCV NS2/3 processing reactions where different fragments are separated on SDS-PAGE. Translated proteins were incubated 60 minutes at 20°C and are identified by arrows adjacent to the lanes. The migration position of molecular weight markers are shown in kDa. Panel A, The NS2/3 reactions shown are: 849-1240J at the start of a 20°C incubation (lane 1), and after 1 hour (lane 2); Mal849-1240J at the start of incubation (lane 3), and after 1 hour (lane 4). Panel B, A representative gel image is shown for the testing of peptides against the 810-1615BK autocleavage. The samples analyzed are: No added peptide (lane 1); peptides of SEQ. ID. NO. 1 (lane 2), SEQ. ID. NO. 2 (lane 3), SEQ. ID. NO. 3 (lane 4), SEQ. ID. NO. 11 (lane 5), SEQ. ID. NO. 4 (lane 6), SEQ. ID. NO. 5 (lane 7), SEQ. ID. NO. 6 (lane 8), DMSO control with no incubation (lane 9). Table 2, *infra*, provides the sequences for the SEQ. ID. NOs.

Figure 2 illustrates titration of NS4A peptide inhibition of NS2/3. Data shown are for peptides of SEQ. ID. NO. 11 (circles) and SEQ. ID. NO. 12 (triangles). IC₅₀ curves are shown after optimization of the adjustable parameters which produced slope coefficients (d) of 1.0 - 1.5 for all fits. IC₅₀ values are in Table 2.

DETAILED DESCRIPTION OF THE INVENTION

The present application identifies the NS4A binding site present on NS2/3 as a target site for inhibiting NS2/3 protease activity. Without being limited to any particular theory, inhibition of NS2/3 by NS4A peptides is believed to be brought about by NS4A acting at the N-terminus of NS3 (the NS2/3 cleavage point). Compounds targeting the HCV target site can be produced independent of such a theory based upon the structure of polypeptides identified herein inhibiting NS2/3 autocleavage and using the guidance provided herein to obtain proteinaceous or non-proteinaceous compounds inhibiting NS2/3 autocleavage.

The compounds and methods described herein have therapeutic and non-therapeutic applications. Non-therapeutic applications include research related applications, such as providing a tool for stabilizing NS2/3 and studying the effects of NS2/3 on HCV polyprotein processing, and for studying the cellular effects of inhibiting NS2/3 autocleavage.

Therapeutic applications include treating a patient infected with HCV and prophylactically treating a patient. Examples of patients that can be infected with HCV include chimpanzees and humans. Prophylactic treatment is preferably performed on patients having a higher risk of being infected with HCV such as those undergoing a blood transfusion.

COMPOUNDS TARGETING THE NS4A TARGET SITE

Using the present application as guide proteinaceous and non-proteinaceous compounds targeting the NS4A target site can be obtained. The provided guidance includes the identification of a target site, examples of compounds directed to the target site, examples of compound modification, and a description of techniques that can be used to obtain additional compounds.

Preferred proteinaceous compounds are those containing a polypeptide region of about 11 contiguous amino acids that bind to the NS4A target site, variants of such compounds, prodrugs of such compounds, and pharmaceutically acceptable

salts thereof. Polypeptide regions of about 11 contiguous amino acids binding to the NS4A target site include amino acid sequences that may, or may not, be present in a naturally occurring NS4A polypeptide.

A variant of a polypeptide refers to a proteinaceous compound containing one or more non-peptide groups. Examples of variants include cyclized peptide analogs, altered amino acid side chains, altered peptide linkages, and the presence of non-amino acid groups. (E.g., Gilon *et al.*, U.S. Patent 5,874,529, and Gante, *Angew. Chem. Int. Ed. Engl.* (1994) 33, 1699-1720, both of which are hereby incorporated by reference herein.)

A prodrug is a substance that is acted on *in vivo* or inside a cell to produce an active compound. The prodrug itself may be active or inactive. Preferably, prodrugs are used to achieve a particular purpose such as facilitating intracellular transport of a compound targeting the NS4A target site. The production of prodrugs facilitating compound intracellular transport is well known in the art, and an example of the production of prodrugs is described by Janmey, *et al.*, U.S. Patent No. 5,846,743, hereby incorporated by reference herein.

Compounds of the present invention include those having one or more chiral centers. The present invention is meant to comprehend diastereomers as well as their racemic and resolved, enantiomerically pure forms and pharmaceutically acceptable salts thereof. Proteinaceous compounds can contain D-amino acids, L-amino acids or a combination thereof. Preferably, amino acids within a chiral center are L-amino acids.

Proteinaceous Compounds

Proteinaceous compounds targeting the NS4A target site contain a polypeptide region targeting the target site. Polypeptide regions targeting the NS4A target site are present at approximately amino acids 22-32 of NS4A. Examples of proteinaceous compounds targeting the NS4A target site are provided for by polypeptide regions found in different NS4A isolates located at amino acids 22-32 of NS4A.

The amino acids sequences located at amino acids 22-32 of NS4A from different isolates of NS4A are well known in the art and can be found in different sources including publications and Gen-Bank. Table 1 provides an example of several NS4A sequences present in different HCV isolates.

Table 1

Isolate	Amino Acid Sequence
HCV-BK	GSVVIVGRILSG (SEQ. ID. NO. 20)
HCV-H ^{1,2}	GCVVIVGRIVLSG (SEQ. ID. NO. 21)
GO1 ³	GSVVIVGRIVLSG (SEQ. ID. NO. 22)
HCV-I ³	GCVVIVGRVVLSG (SEQ. ID. NO. 23)
HCV-J6 ³	GCVCIIGRLHVNQ (SEQ. ID. NO. 24)
HCV-J8 ³	GCISIIGRLHLNQ (SEQ. ID. NO. 25)
HCV-NZL1 ¹	-CVVIVGHIEIEGK (SEQ. ID. NO. 26)

The provided amino acid sequence starts at amino acid 21 of NS4A unless a "-" is present, in which case the amino acid sequence starts at amino acid 22.

¹Butkiewicz, *et al.*, (1996) *Virology* 225, 328-338, hereby incorporated by reference herein.

²Lin, *et al.*, (1995) *J. Virol.* 69, 4373-4380, hereby incorporated by reference herein.

³Bartenshlager, *et al.*, (1995) *J. Virol.* 69, 7519-7528, hereby incorporated by reference herein.

Proteinaceous compounds targeting the NS4A target site can be produced to contain a region corresponding to about amino acids 22-32 of NS4A and can contain additional polypeptide and non-polypeptide regions. The NS4A polypeptide regions are at least about 11 amino acids in length. In different embodiments the NS4A region is at least about 12, 14, 20, 40 amino acids in length.

Additional polypeptide regions that can be present include additional NS4A regions and polypeptide regions or amino acids not related to NS4A. In different embodiments the overall size of the polypeptide is not greater than about 650 amino acids, not greater than about 200 amino acids, not greater than about 100 amino acids, or not greater than about 50 amino acids.

Additional non-polypeptide regions include the presence of amino- and/or carboxy-terminal groups that facilitate cellular uptake and/or facilitate survival of the polypeptide. Possible groups include those cleaved inside a cell and those remaining part of the active compound.

A large number of additional NS4A regions can be selected based upon the known structures of NS4A from different isolates and can be selected independent of the known structures of NS4A. Additional regions selected independent of the known structures of NS4A could be chosen, for example, randomly or to achieve a particular purpose such as producing a prodrug. The affect of additional sequences on

NS2/3 autocleavage can readily be tested using techniques exemplified in the examples provided below.

Polypeptide regions targeting the NS4A target site can also be produced based upon a comparison of NS4A occurring in different isolates and the use of conservative amino acid substitutions. Conservative amino acid substitutions generally involve exchanging amino acids within the same group (*e.g.*, neutral and hydrophobic, neutral and polar, basic, and acidic). Additional amino acid substitutions can readily be identified by testing the effect of different amino acid substitutions on the ability to inhibit NS2/3 autocleavage.

Structure I provides a generic structure of a polypeptide encompassing a region targeting the NS4A target site. Structure I is as follows:



- wherein X^1 is either serine, cysteine, or threonine, preferably serine or cysteine;
 X^2 is either valine, leucine, or isoleucine, preferably valine or isoleucine, more preferably valine;
 X^3 is either valine, leucine, isoleucine, serine, cysteine or threonine, preferably valine or isoleucine, more preferably valine;
 X^4 is either valine, leucine, or isoleucine, preferably valine or isoleucine, more preferably isoleucine;
 X^5 is either valine, leucine, or isoleucine, preferably valine or isoleucine, more preferably valine;
 X^6 is either lysine, arginine, or histidine, preferably arginine or histidine, more preferably arginine;
 X^7 is either valine, leucine, or isoleucine, preferably isoleucine;
 X^8 is either aspartic acid, glutamic acid, valine, leucine, isoleucine, lysine, arginine, or histidine, preferably glutamic acid, valine, leucine, isoleucine, or histidine, more preferably valine, leucine, or isoleucine, more preferably valine;
 X^9 is either valine, leucine, or isoleucine, preferably leucine;
 X^{10} is either serine, cysteine, threonine, asparagine, glutamine, aspartic acid, or glutamic acid, preferably serine, asparagine, or glutamic acid;
each Y^1 is an independently selected amino acid,
each Y^2 is an independently selected amino acid,

Z¹ is an optionally present protecting group covalently joined to Y¹, preferably, Z¹ is either an optionally substituted -C₁₋₁₀ alkyl, optionally substituted -C₂₋₁₀ alkenyl, optionally substituted aryl, -C₁₋₆ alkyl optionally substituted aryl, -C(O)-(CH₂)₁₋₆-COOH, -C(O)-C₁₋₆ alkyl, -C(O)-optionally substituted aryl, -C(O)-O-C₁₋₆ alkyl, or C(O)-O-optionally substituted aryl, more preferably acetyl, propyl, succinyl, benzyl, benzyloxycarbonyl or *t*-butyloxycarbonyl;

Z² is an optionally present protecting group covalently joined to Y², preferably amide, methylamide, or ethylamide;

m is from 0 to 300, in different embodiments m is 0 to 100, 0 to 50, 0 to 25, 0 to 10, and 0 to 5; and

n is from 0 to 300, in different embodiments m is 0 to 100, 0 to 50, 0 to 25, 0 to 10, and 0 to 5.

An "optionally present protecting group covalently joined to Y¹" refers to the presence of a group joined to the amino terminus which reduces the reactivity of the amino terminus under *in vivo* conditions. In the absence of the protecting group -NH₂ is present at the amino terminus.

An "optionally present protecting group covalently joined to Y²" refers to the presence of a group joined to the carboxy terminus which reduces the reactivity of the carboxy terminus under *in vivo* conditions. The carboxy terminus protecting group is preferably attached to the α -carbonyl group of the last amino acid. In the absence of the protecting group -COOH is present at the carboxy terminus.

"Alkyl" refers to carbon atoms joined by carbon-carbon single bonds. The alkyl hydrocarbon group may be straight-chain or contain one or more branches or cyclic groups. Preferably, the alkyl group is 1 to 4 carbons in length. Examples of alkyl include methyl, ethyl, propyl, isopropyl, cyclopropyl, butyl, and *t*-butyl. Alkyl substituents are selected from the group consisting of halogen (preferably -F or -Cl) -OH, -CN, -SH, -NH₂, -NO₂, -C₁₋₂ alkyl substituted with 1 to 6 halogens (preferably -F or -Cl, more preferably -F), -CF₃, -OCH₃, or -OCF₃.

"Alkenyl" refers to a hydrocarbon group containing one or more carbon-carbon double bonds. The alkenyl hydrocarbon group may be straight-chain or contain one or more branches or cyclic groups. Preferably, the alkenyl group is 2 to 4 carbons in length. Alkenyl substituents are selected from the group consisting of halogen (preferably -F or -Cl), -OH, -CN, -SH, -NH₂, -NO₂, -C₁₋₂ alkyl substituted

with 1 to 5 halogens (preferably -F or -Cl, more preferably -F), -CF₃, -OCH₃, or -OCF₃.

"Aryl" refers to an optionally substituted aromatic group with at least one ring having a conjugated pi-electron system, containing up to two conjugated or fused ring systems. Aryl includes carbocyclic aryl, heterocyclic aryl and biaryl groups. Preferably, the aryl is a 5 or 6 membered ring, more preferably benzyl. Aryl substituents are selected from the group consisting of -C₁₋₄ alkyl, -C₁₋₄ alkoxy, halogen (preferably -F or -Cl), -OH, -CN, -SH, -NH₂, -NO₂, -C₁₋₂ alkyl substituted with 1 to 5 halogens (preferably -F or -Cl, more preferably -F), -CF₃, or -OCF₃.

Proteinaceous compounds can be produced using standard techniques. The polypeptide region of a proteinaceous compound, and proteinaceous compounds that are exclusively polypeptide, can be chemically or biochemically synthesized. Techniques for chemical synthesis of polypeptides are well known in the art. (See *e.g.*, Vincent, in *Peptide and Protein Drug Delivery*, New York, N.Y., Dekker, 1990.)

Biochemical production of polypeptides can be performed using cells as biological factories to produce nucleic acid encoding for the polypeptide. Nucleic acid sequences encoding for polypeptides targeting the NS4A target site can be produced by taking into account the genetic code. The genetic code providing the sequences of nucleic acid triplets coding for particular amino acids is well known in the art. Amino acids are encoded for by codons as follows:

A=Ala=Alanine: codons GCA, GCC, GCG, GCU

C=Cys=Cysteine: codons UGC, UGU

D=Asp=Aspartic acid: codons GAC, GAU

E=Glu=Glutamic acid: codons GAA, GAG

F=Phe=Phenylalanine: codons UUC, UUU

G=Gly=Glycine: codons GGA, GGC, GGG, GGU

H=His=Histidine: codons CAC, CAU

I=Ile=Isoleucine: codons AUA, AUC, AUU

K=Lys=Lysine: codons AAA, AAG

L=Leu=Leucine: codons UUA, UUG, CUA, CUC, CUG, CUU

M=Met=Methionine: codon AUG

N=Asn=Asparagine: codons AAC, AAU

P=Pro=Proline: codons CCA, CCC, CCG, CCU

Q=Gln=Glutamine: codons CAA, CAG

R=Arg=Arginine: codons AGA, AGG, CGA, CGC, CGG, CGU

S=Ser=Serine: codons AGC, AGU, UCA, UCC, UCG, UCU

T=Thr=Threonine: codons ACA, ACC, ACG, ACU

V=Val=Valine: codons GUA, GUC, GUG, GUU

5 W=Trp=Tryptophan: codon UGG

Y=Tyr=Tyrosine: codons UAC, UAU

A desired polypeptide may be recombinantly expressed using an expression vector encoding for the desired polypeptide and containing a promoter and other appropriate regulatory elements suitable for transcription and translation of the
10 nucleic acid in a desired host. Expression vectors may be introduced into host cells using standard techniques. Examples of techniques for introducing nucleic acid into a cell and expression of nucleic acids are provided in Ausubel, *Current Protocols in Molecular Biology*, John Wiley, 1987-1998, and Sambrook, *et al.*, in *Molecular Cloning, A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press,
15 1989.

Preferably, expression is achieved in a host cell using an expression vector. An expression vector contains nucleic acid encoding for a desired polypeptide along with regulatory elements for proper transcription and processing. Generally, the regulatory elements that are present include a transcriptional promoter, a ribosome
20 binding site, a terminator, and an optionally present operator. Another preferred element is a polyadenylation signal providing for processing in eukaryotic cells.

Regulatory systems are available to control gene expression, such as GENE-SWITCH™ (Wang, *et al.*, *Gene Ther.* (1997) 4, 432-41, U.S. Patent No. 5,874,534 and International Publication WO 93/23431, each of which are hereby
25 incorporated by reference herein) and those involving the tetracycline operator (U.S. Patent Nos. 5,464,758 and 5,650,298, both of which are hereby incorporated by reference herein).

The skilled artisan can readily identify expression vectors providing suitable levels of polypeptide expression in different hosts. A variety of mammalian
30 expression vectors are well known in the art including pcDNA3 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and .lambda.ZD35 (ATCC 37565). A variety of
35 bacterial expression vectors are well known in the art including pET11a (Novagen),

lambda gt11 (Invitrogen), pcDNAII (Invitrogen), pKK223-3 (Pharmacia). A variety of fungal cell expression vectors are well known in the art including pYES2 (Invitrogen), and Pichia expression vector (Invitrogen). A variety of insect cell expression vectors are well known in the art including Blue Bac III (Invitrogen).

5 Recombinant host cells may be prokaryotic or eukaryotic. Examples of recombinant host cells include the following: bacteria such as *E. coli*; fungal cells such as yeast; mammalian cells such as human, bovine, porcine, monkey and rodent; and insect cells such as *Drosophila* and silkworm derived cell lines. Commercially available mammalian cell lines include L cells L-M(TK.sup.-) (ATCC CCL 1.3), L
10 cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171).

15 A desired polypeptide can be purified by standard techniques such as those using antibodies binding to the polypeptide. Antibodies specifically recognizing a polypeptide can be produced using the polypeptide as an immunogen. Preferably, the polypeptide used as an immunogen should be at least 9 amino acids in length. Examples of techniques for producing and using antibodies are described in Ausubel,
20 *Current Protocols in Molecular Biology*, John Wiley, 1987-1998, Harlow, *et al.*, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988, and Kohler, *et al.*, *Nature* 256:495-497 (1975).

Non-Proteinaceous Compounds

25 Non-proteinaceous compounds targeting the NS4A target site include compounds that are designed based on the structure of polypeptides targeting the NS4A target site and compounds that are selected based on the ability to bind to the NS4A target site.

30 Compounds designed based on the structure of polypeptides targeting the NS4A target site are peptidomimetic compounds. Preferred peptidomimetic compounds have additional characteristics, compared to polypeptides, that enhance their therapeutic applications. Such additional characteristics may include increased cell permeability and prolonged biological half-time. Techniques for designing and synthesizing peptidomimetics are well known in the art. (See, Gilon, *et al.*, U.S.

Patent 5,874,529, and Gante, *Angew. Chem. Int. Ed. Engl.* (1994) 33, 1699-1720, both of which are hereby incorporated by reference herein.)

GENE THERAPY

5 Gene therapy using a nucleic acid encoding for a polypeptide targeting the NS4A target site can be performed taking into account the present disclosure and general gene therapy techniques well known in the art. Preferably, gene therapy is performed using an expression vector.

10 Expression vectors useful in gene therapy include those serving as delivery vehicles and those that are introduced into a cell by a delivery vehicle or appropriate technique. Expression vectors that can act as delivery vehicles are well known in the art, examples of which include retrovirus vectors, adenovirus vectors, and adeno-associated virus vectors. (*Gene Therapy & Molecular Biology: From Basic Mechanisms to Clinical Applications*, Ed. Bouliskas, Gene Therapy Press, 1998, and Hitt, *et al.* (1996) *Advances in Pharmacology* 40:137-206, hereby incorporated by
15 reference herein.) Nonviral gene delivery methods are also well known in the art, examples of which include the use of liposomes, direct injection of DNA and polymers. (*Gene Therapy & Molecular Biology: From Basic Mechanisms to Clinical Applications*, Ed. Bouliskas, Gene Therapy Press, 1998 hereby incorporated by
20 reference herein.)

 Gene therapy can be performed *in vivo* or *ex vivo*. *In vivo* gene therapy is performed by directly administering nucleic acid to a patient. *Ex vivo* gene therapy is performed by administering nucleic acid to cells outside of a patient and then introducing the treated cells into a patient.

25

COMPOUND SCREENING

 The guidance provided herein can be used in methods screening for compounds that inhibit HCV replication or HCV polyprotein processing. Such methods include those identifying HCV inhibitory compounds targeting the NS4A
30 target site and those using NS4A agonists.

 The effect of a compound on HCV polyprotein processing can be tested for by measuring the ability of the compound to alter the formation or activity of products normally produced by HCV polypeptide processing. Preferably, HCV processing is tested for by measuring the activity or formation of NS2 or NS3.

35

Targeting the NS4A Target Site

HCV inhibitory compounds targeting the NS4A target site can be screened for by first identifying a compound that binds to the NS4A target site using a polypeptide comprising NS2/3 or a binding portion thereof. The identified compound
5 is then tested for its ability to inhibit HCV replication or HCV polyprotein processing.

The NS2/3 portion used in the screening contains a sufficient amount of a NS2 region and a NS3 region to bind NS4A. The NS2 region preferably contains at least about 70 amino acids from the NS2 carboxy terminus; and in different
10 embodiments contains at least about 100 or 200 amino acids of NS2. The NS3 region preferably contains at least about 150 amino acids from the amino terminus of NS3; and in different embodiments contains at least about 200 or 300 amino acids.

Compounds binding to the NS4A target site are preferably identified using a competitive assay involving a compound known to bind to the NS4A target site. Such identification may be performed starting with a compound present in a test
15 preparation containing a plurality of different compounds or on a compound by compound basis. Examples of plurality of different compounds include a preparation containing 2 or more, 5 or more, 10 or more, or 20 or more compounds.

Screening in the Presence of an NS4A Agonist

20 Non-saturating levels of an NS4A agonist can be employed in assays screening for HCV inhibitory compounds. Without being limited to any particular theory, NS4A agonists may alter NS2/3 conformation thereby increasing the accessibility of the NS2/3 active site to HCV inhibitory compounds. However, using the guidance provided herein HCV inhibitory compounds can be identified
25 independent of such a theory including HCV inhibitory compounds that bind to an allosteric site in the presence of NS4A.

The NS4A agonist can compete with NS4A for binding to NS2/3 under the conditions used in the screening method. Examples of NS4A agonists include proteinaceous compounds such as NS4A itself, and the peptides described in the
30 examples below. Additional NS4A agonists, including non-proteinaceous compounds, can be identified using the procedures described herein.

Preferably, the NS4A agonist employed in the assay is present at a level sufficient to cause a detectable inhibition of NS2/3 autocleavage. In different
35 embodiments the NS4A agonist is present at a concentration no more than 2X or 1X its K_d or IC_{50} ; or is present at a concentration about equal to its K_d or IC_{50} .

ADMINISTRATION

Compounds targeting the NS4A target site can be formulated and administered to a patient using the guidance provided herein along with techniques well known in the art. The preferred route of administration ensures that an effective amount of compound reaches the target. Guidelines for pharmaceutical administration in general are provided in, for example, *Remington's Pharmaceutical Sciences 18th Edition*, Ed. Gennaro, Mack Publishing, 1990, and *Modern Pharmaceutics 2nd Edition*, Eds. Banker and Rhodes, Marcel Dekker, Inc., 1990, both of which are hereby incorporated by reference herein.

Compounds targeting the NS4A target site having appropriate functional groups can be prepared as acidic or base salts. Pharmaceutically acceptable salts (in the form of water- or oil-soluble or dispersible products) include conventional non-toxic salts or the quaternary ammonium salts that are formed, *e.g.*, from inorganic or organic acids or bases. Examples of such salts include acid addition salts such as acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, and undecanoate; and base salts such as ammonium salts, alkali metal salts such as sodium and potassium salts, alkaline earth metal salts such as calcium and magnesium salts, salts with organic bases such as dicyclohexylamine salts, N-methyl-D-glucamine, and salts with amino acids such as arginine and lysine.

Compounds targeting the NS4A target site can be administered using different routes including oral, nasal, by injection, transdermal, and transmucosally. Active ingredients to be administered orally as a suspension can be prepared according to techniques well known in the art of pharmaceutical formulation and may contain microcrystalline cellulose for imparting bulk, alginic acid or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and sweeteners/flavoring agents. As immediate release tablets, these compositions may contain microcrystalline cellulose, dicalcium phosphate, starch, magnesium stearate

and lactose and/or other excipients, binders, extenders, disintegrants, diluents and lubricants.

When administered by nasal aerosol or inhalation, compositions can be prepared according to techniques well known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents.

The compounds may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. When administered by injection, the injectable solutions or suspensions may be formulated using suitable non-toxic, parenterally-acceptable diluents or solvents, such as Ringer's solution or isotonic sodium chloride solution, or suitable dispersing or wetting and suspending agents, such as sterile, bland, fixed oils, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

When rectally administered in the form of suppositories, these compositions may be prepared by mixing the drug with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters or polyethylene glycols, which are solid at ordinary temperatures, but liquidify and/or dissolve in the rectal cavity to release the drug.

Different techniques and formulations can be used to facilitate introduction of a peptide into a cell, including nucleic acid delivery, prodrug formulations, and liposomes. Examples of such techniques and formulations are described above and in references such as *Gene Therapy & Molecular Biology: From Basic Mechanisms to Clinical Applications*, Ed. Boulikas, Gene Therapy Press, 1998.

Suitable dosing regimens for the therapeutic applications of the present invention are selected taking into factors well known in the art including age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound employed.

Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug. The daily dose for a patient is expected to be between 0.01 and 1,000 mg per adult patient per day.

EXAMPLES

5 Examples are provided below to further illustrate different features and advantages of the present invention. The examples also illustrate useful methodology for practicing the invention. These examples do not limit the claimed invention.

Example 1: Materials and Methods

10 *DNA constructions.* Two DNA constructs were made for the synthesis of NS2/3 J strain RNA, and its subsequent translation to proteins which lack the membrane binding region of NS2 but contain HCV residues 849-1240; "849-1240J" and "Mal849-1240J". Codons 849 to 1240 were amplified by PCR from pT7 (Santolini, *et al.*, (1995) *J. Virol.* 69, 7461-7471). For 849-1240J, the HCV sequence
15 was cloned into pET3c (Novagen), while for Mal849-1240J, the DNA was inserted into pETMalchH (Pryor, *et al.*, (1997) *Protein Expr. Purif.* 10, 309-319) to produce an open reading frame encoding the fusion protein, "Mal849-1240", which includes E. coli maltose binding protein-His₆ tag-HCV residues 849-1240 (Love, *et al.*, (1996) *Cell* 87, 331-342). DNA for pCITE 810-1615BK is described by Pieroni, *et al.*,
20 (1997) *J. Virol.* 71, 6373-80. Upon transcription and translation, pCITE 810-1615BK produces HCV residues 810-1615 of the BK strain ("810-1615BK").

Site-directed mutagenesis was performed with the Stratagene Quick Change method, to generate non-processing mutants His952Ala and Cys993Ala in the expression constructs described above.

25 *Peptides.* Peptides were obtained by custom synthesis from Midwest Biotech (Fishers, IN) and were greater than 95% pure as judged from reverse phase HPLC. Effective molecular weights were obtained by quantitative amino acid analysis. All peptides were dissolved and diluted in DMSO, so that the final concentration of DMSO in every reaction was 5%.

30 *In-vitro transcription and translation.* Circular DNA plasmids were linearized with BLP1 (Bpu 1102) and purified with a Qiagen QiaEX II kit before transcription. RNA was transcribed with T7 RNA polymerase (Ambion Megascript kit), phenol/CHCl₃ extracted and ethanol precipitated. Translations were with Promega or Ambion in-vitro rabbit reticulocyte lysate translation kits at 30°C, for 30-
35 40 minutes using ³⁵S-methionine as a label (NEN, Amersham). Translation was then

inhibited by the addition of cycloheximide (250 μ M final concentration) and samples immediately frozen on dry ice.

5 *NS2/3 autocleavage reactions.* Translated NS2/3 was thawed on ice and cleavage was initiated by incubation at 20°C, either in the original translation
mixture or following a 10-fold dilution into a 10,000 molecular weight filtrate of
reticulocyte lysate produced with Amicon Microcon-10 filters. Samples taken at the
times indicated were combined with SDS gel sample buffer and frozen on dry ice.
NS2/3 cleavage reactions with the 810-1615BK were performed with 1% Triton X-
100 present, as described by Pieroni, *et al.*, (1997) *J. Virol.* 71, 6373-6380. At the
10 completion of an experiment, frozen samples were placed in boiling water for 5
minutes, and radiolabeled proteins were separated by SDS-PAGE (14%). Prestained
Novex molecular weight standards were used in estimation of molecular weights of
the products. For peptide inhibition measurements, incubations were initiated by the
addition of diluted lysate to a DMSO solution of peptide in a tube held at 20°C.

15 The distribution of 35 S-labeled proteins on dried gels was determined
with a phosphorimager (Molecular Dynamics). Product bands were quantified and
expressed as a proportion of total signal in the gel lane so that variations in gel lane
loading were normalized. The product NS2 from 810-1615 BK was used to generate
data shown for screening of peptides and IC₅₀ calculations, due to its migration on
20 gels in a region with less background than the higher molecular weight products, and
due to the ability to initiate the 810-1615BK reaction with Triton X-100 (Santolini, *et al.*, (1995) *J. Virol.* 69, 7461-7471). The IC₅₀ values were determined by first
expressing the product level found as a fraction of the no-inhibitor control product
level, then fitting the equation

25

$$\text{Fractional Activity} = a + \frac{b}{(1 + x/c)^d}$$

to the data, where a is the minimal level of fractional activity (tending to 0), $a+b$ is the
maximal level (tending to 1), x is the concentration of inhibitor, c is the IC₅₀ and d is a
slope coefficient.

30

Example 2: NS2/3 Processing Reactions

Autocleavage reactions using NS2/3 protein translated in-vitro were used to investigate the inhibitory potential of peptides likely to affect the reaction.

- 5 Peptides representing the cleaved sequence had no effect upon reaction rates and the reaction rate was insensitive to dilution. Both results are consistent with suggestions that the NS2/3 cleavage is an intramolecular reaction.

- Typical NS2/3 processing reactions are shown in Figure 1. The reaction occurred on a time scale of minutes, with the rate and final extent of reaction
10 varying somewhat with the sequence expressed. NS2/3 810-1615BK was cleaved as much as 60% with a 3 hour incubation, and the maltose binding protein fusion, Mal849-1240J, to nearly 100%. In all constructs, the mutations His952Ala or Cys993Ala prevented the appearance of products, as previously reported (Hijikata, *et al.*, (1993) *J. Virol.* 67, 4665-4675; and Grakoui, *et al.*, (1993) *Proc. Natl. Acad. Sci.*
15 *USA* 90, 10583-7).

- Both Mal849-1240J and 810-1615BK were used in subsequent characterization of the NS2/3 reaction and inhibition. The translation of Mal849-1240J gave the expected precursor molecular weight of 80 kDa, but also a smaller protein of 67 kDa (Fig. 1A), possibly due to internal initiation, thus complicating the
20 use of this version of NS2/3 for quantification of processing rates and inhibitor potencies. In contrast, 810-1615BK was produced as a single 80 kDa band that cleaved itself to the expected molecular weight products of 60 kDa (NS3) and 20 kDa (NS2) (Fig. 1B). In addition, 810-1615BK did not begin cleavage until addition to Triton X-100, as has been reported (Pieroni, *et al.*, (1997) *J. Virol.* 71, 6373-6380),
25 thereby allowing reactions to be initiated at will without background cleavage products generated during the translation phase of the experiment.

- Dilution of the NS2/3 precursor 10-fold into water completely prevented the processing reaction (data not shown). Dilution into a 10,000 molecular weight filtrate of rabbit reticulocyte lysate supported the reaction at a rate slightly
30 higher than observed in undiluted lysate. Greater dilution of precursor (up to 40-fold) did not further change the rate of processing. The necessity of low molecular weight cellular component(s) for NS2/3 reactions was previously noted. (See, Pieroni, *et al.*, (1997) *J. Virol.* 71, 6373-6380, hereby incorporated by reference herein.)

For all subsequent measurements, a 10-fold dilution of in-vitro synthesized NS2/3 into lysate filtrate was used. The accumulation of products for 810-1615BK occurred at a rate of 0.04 min^{-1} .

5

Example 3: Peptide Inhibition of NS2/3 Processing

Peptide inhibition of NS2/3 processing was measured using peptides containing the NS2/3 cleavage sequence, peptides targeted to the NS4 target site and peptides not related to the target site. Peptides targeted to the NS4A target site were
10 designed based on the region of NS4A binding to the target site. The results are shown in Table 2.

Table 2
Inhibition of NS2/3 by peptides.

SEQ. ID. NO.	SEQUENCE	INHIBITION %	IC ₅₀ ^a (μM)
NS 2/3 Cleavage Site-Derived Peptides ^b			
1	EQGWRL↓ APITAYS	15	
2	GRGLRLL↓ APITAYS	12	
3	EQGWRL	14	
4	APITAYS	-9	
5	GRGLRLL	2	
6	GWRL↓ APITA	20	
7	APITA	6	
8	GWRL	16	
NS4A-Derived Peptides ^c			
9	KGSVVIVGRILSGRK	61	5.7
10	VRLGSISVIGIVRGKK	-17	137.0
11	Ac-GGSVVIVGRILSGRK	66	3.4
12	GGSVVIVGRILSGRG	66	
13	KKGSVVIVGRILSGRPAIVPRR-NH ₂	95	
14	KKGSVVIVGRILSGRPAIVPDRELLY QEFDE	85	
15	Ac-KGSVVIV-NH ₂	8	
16	Ac-AIILSGR	-12	
17	Ac-RILSGRK	-21	
Unrelated Peptides ^b			
18	GVVNAS.Abu.RLATRR	14	
19	HTYLQASEKFKM	11	

^a IC₅₀ values were determined as described in Example 1, and are an average of two determinations.

^b Percent inhibitions shown for cleavage site and unrelated peptides were obtained with a final peptide concentration of 1 mg/ml, which when expressed as a molar

concentration corresponds to a minimum of 500 μ M for the group of cleavage site peptides.

^cPercent inhibitions shown for NS4A peptides were obtained with a final peptide concentration of 50 μ M.

5 "Ac" refers to acetyl.

NS2/3 reactions with 810-1615BK were performed for 30 minutes, as described in Example 1. The NS2/3 cleavage site-derived peptides of SEQ. ID. NOs. 1 and 2 correspond to HCV amino acids 1020-1033, J strain and BK strain, respectively. Other cleavage site-derived peptides are smaller segments of SEQ. ID. NOs. 1 or 2. The arrow indicates the cleavage point, as determined in the NS2/3 protein. (Grakoui, *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90, 10583-10587, and Komoda, *et al.*, (1994) *Gene* 145, 221-226.) SEQ. ID. NO. 9 represents NS4A residues 21-34, and has lysine residues appended to each end to enhance solubility. SEQ. ID. NO. 10 has the same amino acids as SEQ. ID. NO. 9, but in a random order.

15 Similar results were obtained with NS2/3 Mal849-1240J.

Peptides containing the cleavage site sequence of NS2/3, RLL*API (SEQ. ID. NO. 27) (where * denotes the scissile bond), were tested for effect upon NS2/3 processing in reactions of 30 minutes. No significant effect was observed with a variety of substrate or product-like peptides at a concentration of 500 μ M, as shown in Figure 1 and Table 2.

20

NS4A peptides were examined for their effect upon NS2/3 autocleavage. Significant inhibition was observed, as shown for a peptide of SEQ. ID. NO. 9 in Figure 1B. The inhibition appeared to occur immediately, since no pre-incubation of NS2/3 with peptide was performed before initiation of the reaction.

25 Also, changes in inhibitor potency were not observed using 20 minute or 45 minute incubations.

The inhibition by NS4A peptides could be titrated and typical results are shown in Figure 1. Potencies of 3.4 μ M and 5.7 μ M were obtained for peptides of SEQ. ID. NOs. 9 and 11, respectively. Peptides that represented only a portion of the region known to bind to NS3, such as peptides of SEQ. ID. NOs. 15 and 16, did not inhibit. Similar inhibition patterns were observed with both 810-1615BK and Mal849-1615J. A peptide with the same amino acid composition as SEQ. ID. NO. 9 but with a randomized sequence (of SEQ. ID. NO. 10) was not inhibitory. Peptides unrelated to NS4A or the NS2/3 cleavage site were also not inhibitory.

30

Other embodiments are within the following claims. While several embodiments have been shown and described, various modifications may be made without departing from the spirit and scope of the present invention.

WHAT IS CLAIMED IS:

1. A method of inhibiting HCV replication in an HCV infected cell comprising the step of providing to said cell an effective amount of a compound that inhibits NS2/3 autocleavage.
2. The method of claim 1, wherein said compound is selected from the group consisting of:
 - an HCV inhibitor polypeptide comprising an NS4A fragment at least about 11 amino acids in length, wherein said fragment can inhibit autocleavage of NS2/3;
 - a pharmaceutically acceptable salt of said HCV inhibitor polypeptide; and
 - a prodrug thereof.
3. The method of claim 1, wherein compound is selected from the group consisting of:
 - a polypeptide having the structure:
$$Z^1-Y^1_m-X^1X^2X^3X^4X^5GX^6X^7X^8X^9X^{10}-Y^2_n-Z^2$$

wherein X^1 is either serine, cysteine, or threonine;
 X^2 is either valine, leucine, or isoleucine;
 X^3 is either valine, leucine, isoleucine, serine, cysteine or threonine;
 X^4 is either valine, leucine, or isoleucine;
 X^5 is either valine, leucine, or isoleucine;
 X^6 is either lysine, arginine, or histidine;
 X^7 is either valine, leucine, or isoleucine;
 X^8 is either aspartic acid, glutamic acid, valine, leucine, isoleucine, lysine, arginine, or histidine;
 X^9 is either valine, leucine, or isoleucine;
 X^{10} is either serine, cysteine, threonine, asparagine, glutamine, aspartic acid, or glutamic acid;
 each Y^1 is an independently selected amino acid,
 each Y^2 is an independently selected amino acid,

Z¹ is an optionally present protecting group covalently joined to Y¹,
Z² is an optionally present protecting group covalently joined to Y²,
m is from 0 to 300, and
n is from 0 to 300,

5 a pharmaceutically acceptable salt of said polypeptide; and
a prodrug thereof.

4. The method of claim 3, wherein m is from 0 to 25 and n is from
0 to 25.

10

5. The method of claim 4, wherein said compound is said
polypeptide or a pharmaceutically acceptable salt thereof.

6. The method of claim 1, wherein said compound is selected
15 from the group consisting of:
KGSVVIVGRILSGRK (SEQ. ID. NO. 16),
Ac-GGSVVIVGRILSGRK (SEQ. ID. NO. 18),
GGSVVIVGRILSGRG (SEQ. ID. NO. 19),
KKGSVVIVGRILSGRPAIVPRR-NH₂ (SEQ. ID. NO. 20), and
20 KKGSVVIVGRILSGRPAIVPDRELLYQEFDE (SEQ. ID. NO. 21),
or a pharmaceutically acceptable salt thereof.

7. A method of inhibiting HCV replication in an HCV infected
cell comprising the step of introducing into said cell an effective amount of a nucleic
25 acid comprising a nucleotide sequence encoding for a polypeptide comprising an
NS4A fragment at least about 11 amino acids in length, wherein said fragment
inhibits autocleavage of NS2/3.

8. The method of claim 7, wherein said nucleic acid is an
30 expression vector.

9. A method of inhibiting HCV replication in an HCV infected
cell comprising the step of introducing into said cell an effective amount of a nucleic

acid comprising a nucleotide sequence encoding for a polypeptide having the structure:



5

wherein X^1 is either serine, cysteine, or threonine;

X^2 is either valine, leucine, or isoleucine;

X^3 is either valine, leucine, isoleucine, serine, cysteine or threonine;

X^4 is either valine, leucine, or isoleucine;

10

X^5 is either valine, leucine, or isoleucine;

X^6 is either lysine, arginine, or histidine;

X^7 is either valine, leucine, or isoleucine;

X^8 is either aspartic acid, glutamic acid, valine, leucine, isoleucine, lysine, arginine, or histidine;

15

X^9 is either valine, leucine, or isoleucine;

X^{10} is either serine, cysteine, threonine, asparagine, glutamine, aspartic acid, or glutamic acid;

each Y^1 is an independently selected amino acid;

each Y^2 is an independently selected amino acid;

20

m is from 0 to 300; and

n is from 0 to 300.

10. The method of claim 9, wherein said nucleic acid is an expression vector.

25

11. The method of claim 10, wherein m is from 0 to 25, and n is from 0 to 25.

12. A method of treating a patient for HCV comprising the step of inhibiting NS2/3 autocleavage.

30

13. The method of claim 12, wherein said patient is a human patient and said method further comprises the step of identifying said patient as infected with HCV prior to said inhibiting.

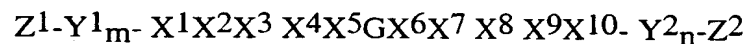
35

14. The method of claim 12, wherein said step of inhibiting NS2/3 autocleavage is achieved using an effective amount of a compound selected from the group consisting of:

- 5 a polypeptide comprising an NS4A fragment at least about 11 amino acids in length;
- a pharmaceutically acceptable salt of said polypeptide; and
- a prodrug thereof.

15. The method of claim 12, wherein said step of inhibiting NS2/3 autocleavage is achieved using an effective amount of a compound selected from the group consisting of:

a polypeptide having the structure:



15

wherein X^1 is either serine, cysteine, or threonine;

X^2 is either valine, leucine, or isoleucine;

X^3 is either valine, leucine, isoleucine, serine, cysteine or threonine;

X^4 is either valine, leucine, or isoleucine;

20 X^5 is either valine, leucine, or isoleucine;

X^6 is either lysine, arginine, or histidine;

X^7 is either valine, leucine, or isoleucine;

X^8 is either aspartic acid, glutamic acid, valine, leucine, isoleucine, lysine, arginine, or histidine;

25 X^9 is either valine, leucine, or isoleucine;

X^{10} is either serine, cysteine, threonine, asparagine, glutamine, aspartic acid, or glutamic acid;

each Y^1 is an independently selected amino acid,

each Y^2 is an independently selected amino acid,

30 Z^1 is an optionally present protecting group covalently joined to Y^1 ,

Z^2 is an optionally present protecting group covalently joined to Y^2 ,

m is from 0 to 300, and

n is from 0 to 300,

35

a pharmaceutically acceptable salt of said polypeptide; and
a prodrug thereof.

16. A method of inhibiting or preventing HCV replication in a patient comprising the step of treating said patient with an effective amount of a compound selected from the group consisting of:

- 5 a polypeptide that either comprises an NS4A fragment at least about 11 amino acids in length able to inhibit NS2/3 autocleavage or has the structure:



- 10 wherein X^1 is either serine, cysteine, or threonine;
 X^2 is either valine, leucine, or isoleucine;
 X^3 is either valine, leucine, isoleucine, serine, cysteine or threonine;
 X^4 is either valine, leucine, or isoleucine;
 X^5 is either valine, leucine, or isoleucine;
15 X^6 is either lysine, arginine, or histidine;
 X^7 is either valine, leucine, or isoleucine;
 X^8 is either aspartic acid, glutamic acid, valine, leucine, isoleucine, lysine, arginine, or histidine;
 X^9 is either valine, leucine, or isoleucine;
20 X^{10} is either serine, cysteine, threonine, asparagine, glutamine, aspartic acid, or glutamic acid;
each Y^1 is an independently selected amino acid,
each Y^2 is an independently selected amino acid,
 Z^1 is an optionally present protecting group covalently joined to Y^1 ,
25 Z^2 is an optionally present protecting group covalently joined to Y^2 ,
m is from 0 to 300, and
n is from 0 to 300,

a pharmaceutically acceptable salt of said polypeptide; and
a prodrug thereof.

30

17. The method of claim 16, wherein said patient is a human infected with HCV.

18. The method of claim 17, wherein said compound is said
35 polypeptide or a pharmaceutically acceptable salt thereof.

19. The method of claim 17, wherein said compound is selected from the group consisting of:

KGSVVIVGRILSGRK (SEQ. ID. NO. 16),
 5 Ac-GGSVVIVGRILSGRK (SEQ. ID. NO. 18),
 GGSVVIVGRILSGRG (SEQ. ID. NO. 19),
 KKGSVVIVGRILSGRPAIVPRR-NH₂ (SEQ. ID. NO. 20), and
 KKGSVVIVGRILSGRPAIVPDRELLYQEFDE (SEQ. ID. NO. 21),
 or a pharmaceutically acceptable salt thereof.

10

20. A method of inhibiting or preventing HCV replication in a patient comprising the step of administering to said patient an effective amount of a nucleic acid comprising a nucleotide sequence encoding for a polypeptide comprising an NS4A fragment at least about 11 amino acids in length, wherein said fragment
 15 inhibits autocleavage of NS2/3.

21. The method of claim 20, wherein said nucleic acid is an expression vector.

20

22. A method of inhibiting or preventing HCV replication in a patient comprising the step of administering to said patient an effective amount of a nucleic acid comprising a nucleotide sequence encoding for a polypeptide having the structure:

25

$$Y^1_m - X^1 X^2 X^3 X^4 X^5 G X^6 X^7 X^8 X^9 X^{10} - Y^2_n$$

wherein X¹ is either serine, cysteine, or threonine;

X² is either valine, leucine, or isoleucine;

X³ is either valine, leucine, isoleucine, serine, cysteine or threonine;

30

X⁴ is either valine, leucine, or isoleucine;

X⁵ is either valine, leucine, or isoleucine;

X⁶ is either lysine, arginine, or histidine;

X⁷ is either valine, leucine, or isoleucine;

X⁸ is either aspartic acid, glutamic acid, valine, leucine, isoleucine, lysine, arginine,

35

or histidine;

X⁹ is either valine, leucine, or isoleucine;
 X¹⁰ is either serine, cysteine, threonine, asparagine, glutamine, aspartic acid, or glutamic acid;
 each Y¹ is an independently selected amino acid;
 5 each Y² is an independently selected amino acid;
 m is from 0 to 300; and
 n is from 0 to 300.

23. The method of claim 22, wherein said nucleic acid is an
 10 expression vector.

24. The method of claim 22, wherein m is from 0 to 25 and
 n is from 0 to 25.

25. A compound selected from the group consisting of:
 a pharmaceutically acceptable salt of a HCV inhibitor
 polypeptide, wherein said HCV inhibitor polypeptide comprises an NS4A fragment at
 least about 11 amino acids in length and can inhibit autocleavage of NS2/3; and
 a prodrug thereof.

26. A compound selected from the group consisting of:
 a polypeptide having the structure:



25 wherein X¹ is either serine, cysteine, or threonine;
 X² is either valine, leucine, or isoleucine;
 X³ is either valine, leucine, isoleucine, serine, cysteine or threonine;
 X⁴ is either valine, leucine, or isoleucine;
 30 X⁵ is either valine, leucine, or isoleucine;
 X⁶ is either lysine, arginine, or histidine;
 X⁷ is either valine, leucine, or isoleucine;
 X⁸ is either aspartic acid, glutamic acid, valine, leucine, isoleucine, lysine, arginine,
 or histidine;

- X⁹ is either valine, leucine, or isoleucine;
X¹⁰ is either serine, cysteine, threonine, asparagine, glutamine, aspartic acid, or glutamic acid;
each Y¹ is an independently selected amino acid,
5 each Y² is an independently selected amino acid,
Z¹ is an optionally present protecting group covalently joined to Y¹,
Z² is an optionally present protecting group covalently joined to Y²,
m is from 0 to 300, and
n is from 0 to 300;
10 a pharmaceutically acceptable salt of said polypeptide; and
a prodrug thereof;
provided that if said compound is said polypeptide then at least
one of Z¹ or Z² is present.
- 15 27. The compound of claim 26, wherein m is from 0 to 25, and n is
from 0 to 25.
28. The compound of claim 27, wherein said compound is said
pharmaceutically acceptable salt.
- 20 29. A compound selected from the group consisting of:
KGSVVIVGRILSGRK (SEQ. ID. NO. 16),
Ac-GGSVVIVGRILSGRK (SEQ. ID. NO. 18),
GGSVVIVGRILSGRG (SEQ. ID. NO. 19),
25 KKGSVVIVGRILSGRPAIVPRR-NH₂ (SEQ. ID. NO. 20), and
KKGSVVIVGRILSGRPAIVPDRELLYQEFDE (SEQ. ID. NO. 21),
or a pharmaceutically acceptable salt thereof.
- 30 30. A nucleic acid comprising a nucleotide sequence encoding for
the HCV inhibitor polypeptide of claim 25.
31. The nucleic acid of claim 30, wherein said nucleic acid is an
expression vector.

32. A nucleic acid comprising a nucleotide sequence encoding for the polypeptide of claim 26.

5 33. The nucleic acid of claim 32, wherein said nucleic acid is an expression vector.

34. A pharmaceutical composition for inhibiting HCV replication comprising;
a pharmaceutically acceptable carrier; and
10 an effective amount of a compound selected from the group consisting of:
an HCV inhibitor polypeptide comprising an NS4A fragment at least about 11 amino acids in length, wherein said fragment can inhibit autocleavage of NS2/3;
15 a pharmaceutically acceptable salt of said HCV inhibitor polypeptide; and
a prodrug thereof.

20 35. A pharmaceutical composition for inhibiting HCV replication comprising:
a pharmaceutically acceptable carrier; and
an effective amount of a polypeptide having the structure:



25

wherein X^1 is either serine, cysteine, or threonine;

X^2 is either valine, leucine, or isoleucine;

X^3 is either valine, leucine, isoleucine, serine, cysteine or threonine;

X^4 is either valine, leucine, or isoleucine;

30 X^5 is either valine, leucine, or isoleucine;

X^6 is either lysine, arginine, or histidine;

X^7 is either valine, leucine, or isoleucine;

X^8 is either aspartic acid, glutamic acid, valine, leucine, isoleucine, lysine, arginine, or histidine;

35 X^9 is either valine, leucine, or isoleucine;

X¹⁰ is either serine, cysteine, threonine, asparagine, glutamine, aspartic acid, or glutamic acid;

each Y¹ is an independently selected amino acid,

each Y² is an independently selected amino acid,

5 Z¹ is an optionally present protecting group covalently joined to Y¹,

Z² is an optionally present protecting group covalently joined to Y²,

m is from 0 to 300, and

n is from 0 to 300;

10 a pharmaceutically acceptable salt of said polypeptide; and
a prodrug thereof.

36. A pharmaceutical composition for inhibiting HCV replication comprising: a pharmaceutically acceptable carrier; and an effective amount of a nucleic acid encoding for a polypeptide comprising a fragment of NS4A at least about
15 11 amino acids in length, wherein said fragment can inhibit autocleavage of NS2/3.

37. The composition of claim 36, wherein said nucleic acid is present in an expression vector providing for expression in a human.

20 38. A pharmaceutical composition for inhibiting HCV replication comprising a pharmaceutically acceptable carrier and an effective amount of a nucleic acid encoding for a polypeptide having the structure:



25 wherein X¹ is either serine, cysteine, or threonine;

X² is either valine, leucine, or isoleucine;

X³ is either valine, leucine, isoleucine, serine, cysteine or threonine;

X⁴ is either valine, leucine, or isoleucine;

30 X⁵ is either valine, leucine, or isoleucine;

X⁶ is either lysine, arginine, or histidine;

X⁷ is either valine, leucine, or isoleucine;

X⁸ is either aspartic acid, glutamic acid, valine, leucine, isoleucine, lysine, arginine, or histidine;

X⁹ is either valine, leucine, or isoleucine;

X¹⁰ is either serine, cysteine, threonine, asparagine, glutamine, aspartic acid, or glutamic acid;

each Y¹ is an independently selected amino acid;

5 each Y² is an independently selected amino acid;

m is from 0 to 300; and

n is from 0 to 300.

39. The composition of claim 38, wherein said nucleic acid is
10 present in an expression vector providing for expression in a human.

40. A method for inhibiting HCV polyprotein processing
comprising the step of contacting a cell expressing an HCV polypeptide that contains
at least NS2/3 with an inhibitory polypeptide that either comprises an NS4A fragment
15 at least about 11 amino acids in length able to inhibit NS2/3 autocleavage or has the
structure:



20 wherein X¹ is either serine, cysteine, or threonine;

X² is either valine, leucine, or isoleucine;

X³ is either valine, leucine, isoleucine, serine, cysteine or threonine;

X⁴ is either valine, leucine, or isoleucine;

X⁵ is either valine, leucine, or isoleucine;

25 X⁶ is either lysine, arginine, or histidine;

X⁷ is either valine, leucine, or isoleucine;

X⁸ is either aspartic acid, glutamic acid, valine, leucine, isoleucine, lysine, arginine,
or histidine;

X⁹ is either valine, leucine, or isoleucine;

30 X¹⁰ is either serine, cysteine, threonine, asparagine, glutamine, aspartic acid, or
glutamic acid;

each Y¹ is an independently selected amino acid,

each Y² is an independently selected amino acid,

Z¹ is an optionally present protecting group covalently joined to Y¹,

polypeptide; and
a pharmaceutically acceptable salt of said inhibitory
a prodrug thereof.

a pharmaceutically acceptable salt of said inhibitory

a prodrug thereof.

41. The method of claim 40, wherein said polypeptide is selected from the group consisting of:

KGSVVIVGRILSGRK (SEQ. ID. NO. 16),
Ac-GGSVVIVGRILSGRK (SEQ. ID. NO. 18),
GGSVVIVGRILSGRG (SEQ. ID. NO. 19),
KKGSVVIVGRILSGRPAIVPRR-NH₂ (SEQ. ID. NO. 20), and
KKGSVVIVGRILSGRPAIVPDRELLYQEFDE (SEQ. ID. NO. 21).

a) selecting for a compound that binds to the NS4A target site using a polypeptide comprising NS2/3 or a binding portion thereof, and

b) measuring the ability of said compound to inhibit HCV replication or HCV polypeptide processing.

b) measuring the ability of said compound to inhibit HCV replication or HCV polyprotein processing.

43. The method of claim 42, wherein said method measures the ability of said compound to inhibit HCV polyprotein processing.

45. A method of screening for a compound that inhibits HCV replication or HCV polyprotein processing comprising the step of measuring the ability of said compound to inhibit HCV replication or HCV polyprotein processing in the presence of a non-saturating amount of a NS4A agonist.

replication or HCV polyprotein processing comprising the step of measuring the ability of said compound to inhibit HCV replication or HCV polyprotein processing in the presence of a non-saturating amount of a NS4A agonist.

1/2

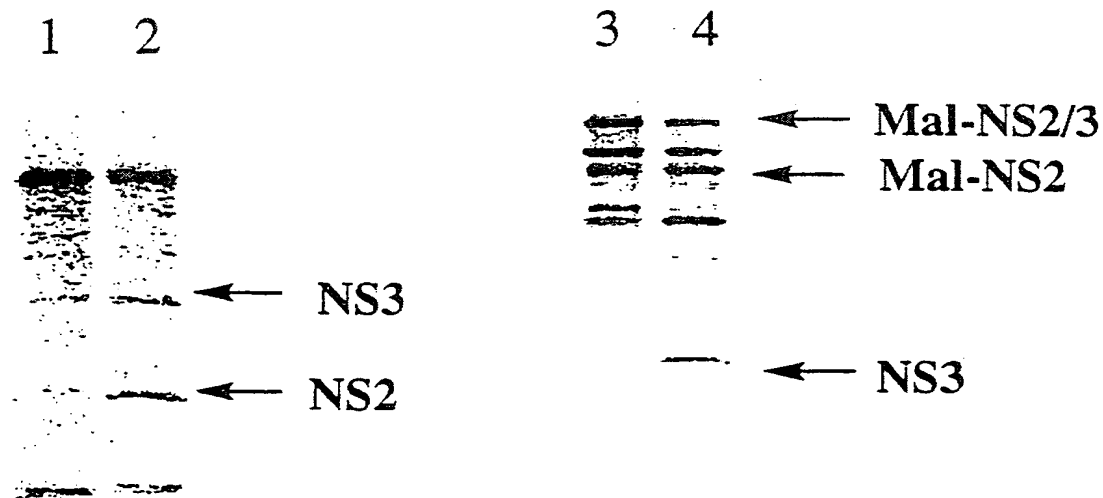


FIG.1A

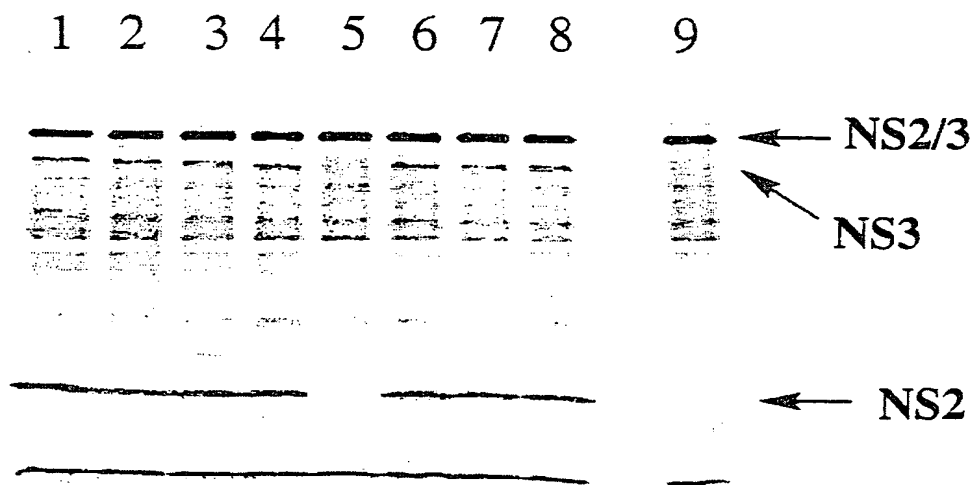


FIG.1B

2/2

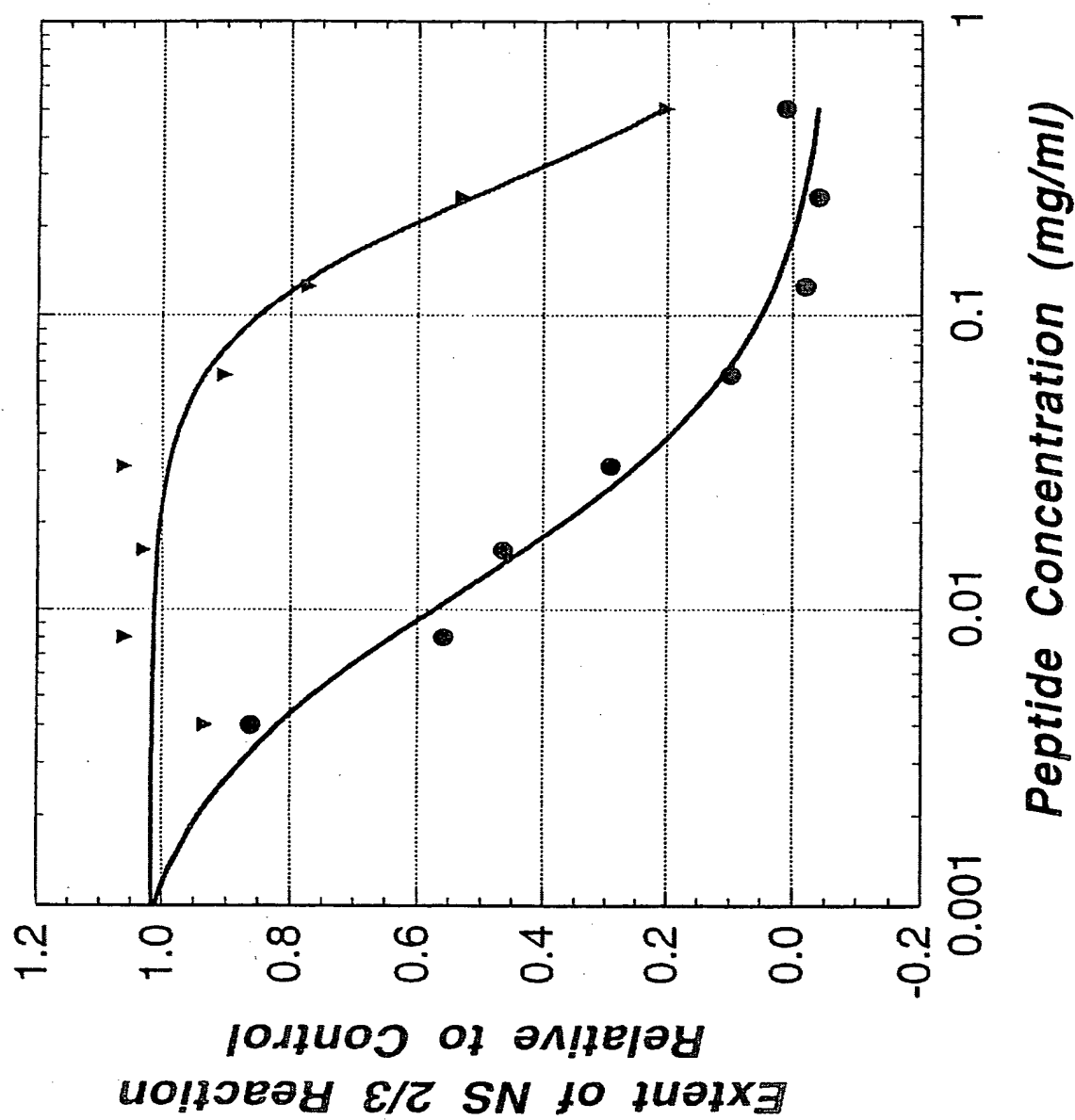


FIG. 2

SEQUENCE LISTING

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Pro	Ala	Ile	Val	Pro	Asp	Arg	Glu	Leu	Leu	Tyr	Gln	Glu	Phe	Asp
			20				25					30		

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<223> NS4A-Derived Peptide

<221> ACETYLTATION

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1				5		

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1				5			

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<223> Cleavage Site Sequence of NS2/3

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Arg Leu Leu Ala Pro Ile
1 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/23444

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/70; A61K 31/711, 38/55; C07K 14/18; C07H 21/04

US CL : 435/5; 514/12, 14, 15, 44; 530/324, 327, 350; 536/ 23.72

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5; 514/12, 14, 15, 44; 530/324, 327, 350; 536/ 23.72

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST: USPT, EPAB, JPAB, DWPI; DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ---- Y,P	DARKE, P.L. et al. Inhibition of Hepatitis C Virus NS2/3 Processing by NS4A Peptides. The Journal of Biological Chemistry. December 1999, Vol. 274, No. 49, pages 34511-34514, see entire document.	1-5,12-18, 25-28,34, 35,40, 42-45 ----- 7-11, 20-24,30-33,36-39
Y	INGALLINELLA, P. et al. Potent Peptide Inhibitors of Human Hepatitis C Virus NS3 Protease Are Obtained by Optimizing the Cleavage Products. Biochemistry. June 1998, Vol. 37, No. 25, pages 8906-8914, see entire document.	1-5,7-18,20 -28, 30-40, 42-45



Further documents are listed in the continuation of Box C.



See patent family annex.

Special categories of cited documents:	
A document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
B earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	*G* document member of the same patent family
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

30 OCTOBER 2000

Date of mailing of the international search report

06 DEC 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Donna C. Wortman
DONNA C. WORTMAN, PH.D.

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/23444

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 6, 19, 29, 41
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

the sequence identification numbers and the amino acid sequences recited in claims 6, 19, 29, and 41 do not correspond to the CRF and the sequence paper copy.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/23444

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LANDRO, J.A. et al. Mechanistic Role of an NS4A Peptide Cofactor with the Truncated NS3 Protease of Hepatitis C Virus: Elucidation of the NS4A Stimulatory Effect via Kinetic Analysis and Inhibitor Mapping. Biochemistry. 1997, Vol. 36, pages 9340-9348, see entire document.	1-5,7-18,20-28,30-40, 42-45
Y,P	US 5,990,276 A (ZHANG et al.) 23 November 1999, see entire document, especially SEQ ID NO.11, see entire document.	1-5,7-18,20-28,30-40,42-45
Y	US 5,767,233 A (ZHANG et al.) / 6 June 1998, see entire document.	1-5,7-18,20-28,30-40,42-45
Y	WO 97/08304 A2 (ISTITUTO DI RICERCHE DI BIOLOGIA MOLECOLARE P. ANGELETTI S.P.A.) 06 March 1997, see entire document.	1-5,7-18,20-28,30-40,4 2-45